Specific interaction between OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and secreted proteins

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OutD is an outer membrane component of the main terminal branch of the general secretory pathway (GSP) in *Erwinia chrysanthemi*. We analyzed the interactions of OutD with other components of the GSP (Out proteins) and with secreted proteins (PelB, EGZ and PemA). OutD is stabilized by its interaction with another GSP component, OutS. The 62 C-terminal amino acids of OutD are necessary for this interaction. *In vivo* formation of OutD multimers, up to tetramers, was proved after the dissociation in mild conditions of the OutD aggregates formed in the outer membrane. Thus, OutD could form a channel-like structure in the outer membrane. We showed that OutD is stabilized *in vivo* when co-expressed with Out-secreted proteins. This stabilization results from the formation of complexes that were detected in experiments of co-immuno-precipitation and co-sedimentation in sucrose density gradients. The presence of the N-terminal part of OutD is required for this interaction. The interaction between OutD and the secreted protein PelB was confirmed *in vitro*, suggesting that no other component of the GSP is required for this recognition. No interaction was observed between the *E.carotovora* PelC and the *E.chrysanthemi* OutD. Thus, the interaction between GspD and the secreted proteins present in the periplasm could be the key to the specificity of the secretion machinery and a trigger for that process.
Keywords: *Erwinia chrysanthemi*/general secretory pathway/GspD/protein–protein interaction/secretion specificity

Introduction

Gram-negative bacteria have developed different pathways in order to secrete proteins in the extracellular medium (Pugsley, 1993; Salmond and Reeves, 1993). In the type I and type III secretion systems, secreted proteins pass directly from the cytoplasm to the outer medium through a specific apparatus. The type I secretion apparatus is composed of three proteins while the type III machinery contains probably >15 proteins. In the type II system, or main terminal branch of the general secretory pathway (MTB of GSP), secretion occurs in two steps: secreted proteins are first translocated by a Sec-dependent mechan-ism into the periplasm, where they can be transiently accumulated (He *et al.*, 1991; Pugsley *et al.*, 1991), their subsequent export across the outer membrane is dependent on the MTB machinery.

A type II secretion system (the Out system) has been identified in *Erwinia chrysanthemi* where it is required for the secretion of several plant cell wall-degrading enzymes: seven pectate lyases (PelA, B, C, D, E, L and Z), the pectin methylesterase PemA and the cellulase EGZ (Ji *et al.*, 1987; Condemine *et al.*, 1992; Lindeberg and Collmer, 1992). Such type II secretion systems have also been found in *Erwinia carotovora*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila*, where they are usually involved in the secretion of virulence factors. They are composed of at least 14 proteins designated by the general name Gsp. Most of them (GspC, E, F, G, H, I, J, K, L, M and O) are cytoplasmic membrane or cytoplasmic membrane-associated proteins (Reeves *et al.*, 1993). The N-terminal region of GspG, H, I and J shows similarity to the type IV pilin subunit. It has been proposed that these proteins could form a pilin-like structure, but the existence of such a structure has never been demonstrated (Pugsley, 1996). GspO is a pre-pilin peptidase that cleaves the signal sequence of GspG, H, I and J (Bally *et al.*, 1992). The GspE sequence contains two Walker boxes, usually present in proteins with an ATPase activity and thus it could provide energy for the secretion process (Possot and Pugsley, 1994; Sandkvist *et al.*, 1995).

Only GspD and GspS are outer membrane proteins. GspS proteins have been identified in *K.oxytoca* (PulS), *E.carotovora* and *E.chrysanthemi* (OutS), but not in other type II secretion systems. PulS is a lipoprotein required for the protection and the insertion of PulD in the outer membrane and thus it plays a chaperone-like role (Hardie *et al.*, 1996). GspD is a member of a large superfamily of proteins involved in the transport of macromolecules across bacterial membranes (Martin *et al.*, 1993; Genin and Boucher, 1994). Besides type II protein secretion systems, GspD homologs are also involved in the type III protein secretion machinery, in exogenous DNA uptake in *Haemophilus influenzae*, in type IV pilus morphogenesis and in filamentous phage release. The proteins of this superfamily have a conserved C-terminal region and a variable N-terminal domain. Nevertheless, the N-terminal domains of proteins involved in the same type of secretion pathway (type II, type III, etc.) show some similarities (Genin and Boucher, 1994). The filamentous phage f1 protein pIV, one of the GspD homologs, was shown to form in the outer membrane, multimers consisting of 10–12 monomers (Kazmierczak *et al.*, 1994). It has been proposed that these complexes constitute pores allowing bacteriophage extrusion. OutD is capable of forming mixed multimers with pIV probably through their conserved

C-terminal regions (Kazmierczak *et al.*, 1994). Thus, the formation of a channel across which secreted proteins could pass has also been suggested for OutD and other GspD proteins.

Until now, the mechanism by which secreted proteins interact with the secretion machinery has remained unknown. However, one important prerequisite for this process is the folding of the proteins in the periplasm into their native, or almost native, state (Hirst and Holmgren, 1987; Pugsley, 1992; Shevchik *et al.*, 1995). The cytoplasmic membrane-associated Gsp proteins are probably not required for the translocation of the secreted proteins into the periplasm since, in their absence, this process occurs via the Sec system (He *et al.*, 1991). The role of most of these cytoplasmic membrane proteins in the translocation of secreted proteins across the outer membrane remains unknown. In contrast, the outer membrane Gsp proteins seem to be at least involved in a channeling function and they could also play a role in the recognition of the secreted proteins. To investigate the precise role of OutD, we have analyzed two properties of this protein: its ability to multimerize in the outer membrane and its ability to interact with Gsp and secreted proteins.

## Results

OutD forms multimers in the outer membrane

The formation of homomultimers by three homologs of OutD (pIV, PulD and XpsD) has been demonstrated by cross-linking experiments (Kazmierczak *et al.*, 1994; Chen *et al.*, 1996; Hardie *et al.*, 1996). PulD and pIV form very stable high molecular weight complexes *in vivo* that are not dissociated by boiling in standard Laemmli sample buffer (SB) (Hardie *et al.*, 1996; Linderoth *et al.*, 1996). Such OutD complexes were also observed when outer membrane fractions of *E.chrysanthemi* or *Escherichia coli* expressing *outD* were loaded, unboiled in SB, onto an SDS–polyacrylamide gel. Progressive heating of the samples led to the disappearance of these complexes and to the appearance of monomeric and oligomeric forms of OutD. Multimers up to tetramers could be detected (Figure 1). The complexes were totally dissociated after boiling of the samples. The complexes formed by OutD seem to be less stable than those formed by PulD or pIV.

OutS is necessary for the stabilization of OutD

Hardie *et al.* (1996) have shown that PulS is required for the stabilization and the insertion of PulD in the outer membrane. We tested whether OutS, the only known homolog of PulS, would play a similar role with OutD. The amount of OutD was estimated by immunoblotting in *E.coli* strains with or without OutS. The presence of OutS led to a drastic increase in the quantity of full size OutD in the bacteria and to a significant decrease in OutD truncated products (Figure 2). However, full size OutD was also detectable in the absence of OutS: in this case, the amount of OutD was dependent on the strain and the growth temperature used (data not shown). OutS could either stabilize OutD or play a role in its insertion in the outer membrane. In that case, insertion would protect OutD from degradation. To distinguish between these two possibilities, fractionation of the membranes of cells expressing *outD* in the presence or absence of *outS* was
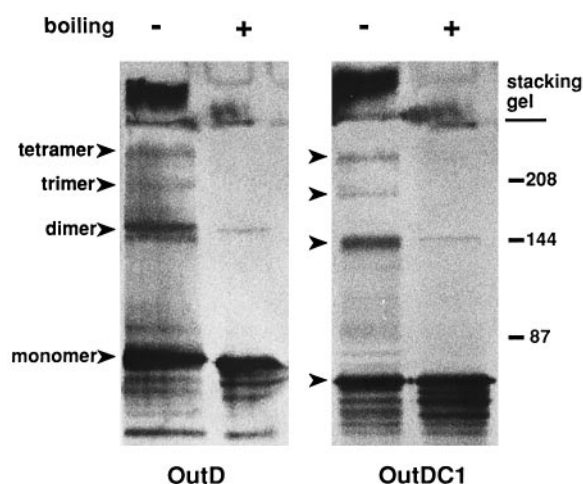
Fig. 1. Identification of OutD homomultimers. The outer membranes of *E.coli* BL21(pTdB-OD) and BL21(pTdB-ODC1) strains, overproducing OutD and OutDC1 respectively, were prepared. The samples (~10 mg of protein/ml) were incubated in a loading buffer containing SDS (0.5%) and β-mercaptoethanol (3%) at 37°C for 15 min or in boiling water for 3 min. Proteins were separated in SDS–PAGE and analyzed by immunoblotting with anti-OutD. The positions of the OutD and OutDC1 homomultimers and the molecular mass standard are indicated.
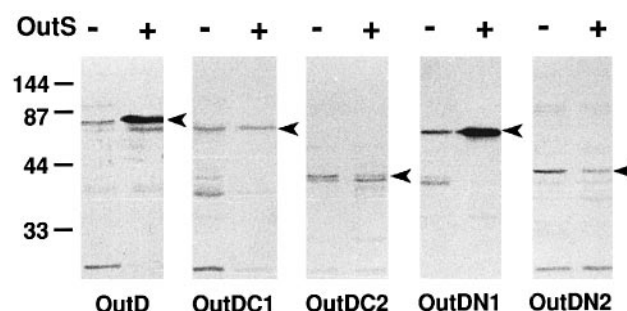

Fig. 2. Stabilization of OutD by OutS *in vivo*. The plasmids coding for OutD and its derivatives (Figure 8) were introduced into *E.coli* NM522 carrying pACOS (OutS+) or pACYC184 (OutS−). The amounts of OutD and its derivatives in the cells were examined by SDS–PAGE and immunoblotting with anti-OutD. The positions of OutD, its derivatives and the molecular mass standard positions are indicated.

performed by flotation in a sucrose gradient. In spite of large differences in the total amount of OutD detectable in the absence or presence of OutS, an almost similar position of OutD on the gradient was observed in both cases: OutD was detected in nearly equal amounts in the outer membrane fraction and in the bottom of the gradient (Figure 3).

Secreted proteins stabilize OutD in vivo

Co-expression in *E.coli* of *pelB* and *outD* at 37°C (but not at 30°C) induced lysis of the bacteria when the OD_{600} reached 0.3–0.5. This effect was not observed when these genes were expressed individually. While bacteria expressing *outS* and *outD* or *outS* and *pelB* were healthy, the bacteriolytic effect was observed when these three genes were co-expressed. Thus, we supposed that secreted proteins can interact with OutD. We checked whether OutD could be protected by the presence of secreted proteins. The amount of OutD was estimated by immunoblotting in *E.coli* strains grown at 30°C, either expressing
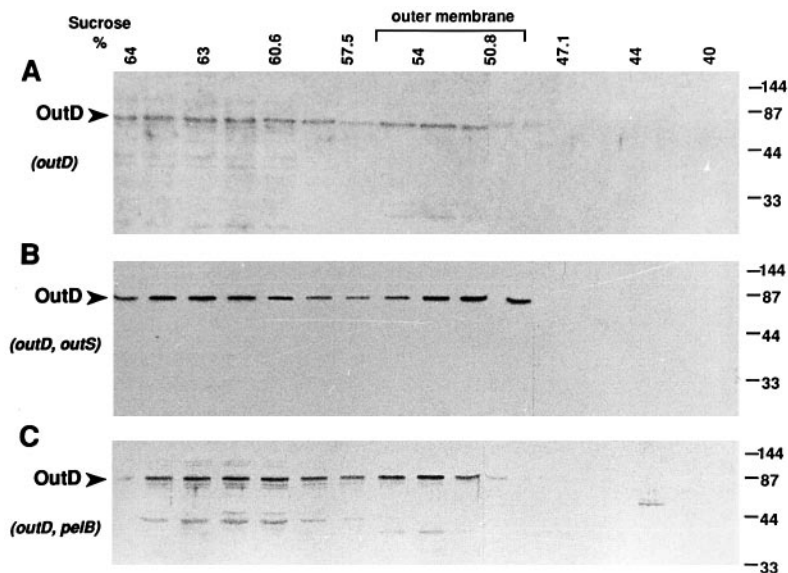
Fig. 3. Effect of OutS and PelB on the OutD cellular localization. Cell lysates of NM522(pTdB-OD) (OutD$^+$) (**A**), NM522(pTPLB-OD/pACOS) (OutD$^+$, OutS$^+$) (**B**) and NM522(pTdB-OD/pACPLB) (OutD$^+$, PelB$^+$) (**C**) were fractionated in flotation sucrose gradient. Aliquots from each fraction were analyzed by SDS–PAGE and immunoblotting with anti-OutD. The nitrocellulose membranes were exposed for 15 (A), 2 (B) and 5 min (C). The sucrose concentrations are indicated.

or not expressing the gene of a secreted protein (Figure 4A). A protection effect with PelB was clearly visible, although less important than the one observed with OutS. The same effect was detected when the *outD* and *pelB* genes were expressed on compatible plasmids (pACPLB and pTdB-OD) or when *pelB* and *outD* were cloned on the same plasmid (pTPLB-OD) (Figure 4A). This protection effect could be reproduced by co-expression of *outD* with the genes of other secreted proteins, *pemA* and *celZ* (Figure 4A). The gene *celZ*, encoding EGZ, was cloned in both orientations in pBluescript. In pSCLZ, *celZ* was under the control of its own promoter, while in pKCLZ it was under the control of the inducible promoter P*lac*, allowing for a higher expression of *celZ*. The quantity of OutD detected in the cells was correlated with the amount of EGZ synthesized, indicating that the protection effect is stoichiometric with the amount of secreted protein (Figure 4B). To exclude the possibility that this protection could result from the overproduction of any protein in the periplasm, OutD was co-expressed with the periplasmic protein MalE. Induction of *malE* expression did not change the level of OutD in the bacteria (Figure 4C). In *E.coli* strains co-expressing *outD* with genes of secreted proteins, the amount of secreted protein also increased, indicating that the protection effect is reciprocal (data not shown).

Both OutS and the secreted proteins are able to protect OutD from degradation. Co-expression in the same strain of *outS* and *pelB* gave a higher level of OutD protection than any of these genes alone (data not shown). The additive effects of OutS and the secreted proteins led us to suppose that they could interact with different parts of OutD.

### PelB interacts with OutD in vivo
To analyze whether the protection of OutD by secreted proteins results from their physical interaction, we tried to detect the formation of a stable complex containing these two proteins. In *E.coli* OutD is located in the outer membrane (Figure 3), while PelB is a periplasmic soluble protein. If secreted proteins are capable of binding OutD, it should be possible to detect PelB in the outer membrane fraction when bacteria produce OutD. We compared the PelB location in *E.coli* strains, either producing or not producing OutD. To increase the amount of OutD, *outS* was also co-expressed. The membrane fractions of NM522(pTPLB) (PelB$^+$) and NM522(pTPLB-OD/pACOS) (PelB$^+$, OutD$^+$, OutS$^+$) were separated by flotation in a sucrose density gradient (Figure 5). In the absence of OutD, PelB was only found in the soluble protein fraction (bottom fractions of the gradient). In the presence of OutD, a portion of PelB co-migrated with the outer membrane fraction. As a control, we verified that the position of alkaline phosphatase, a periplasmic protein, was not affected in the presence of OutD. These results strongly suggest the existence of complexes containing OutD and PelB.

To confirm this result, we performed co-precipitation analysis. The *E.coli* NM522(pTPLB-OD/pACOS) outer membrane fraction containing OutD and PelB was incubated with biotinylated PelB to chase the unlabeled PelB from the complexes. Streptavidin–agarose was added to bind biotinylated PelB. Analysis of the proteins bound to streptavidin–agarose revealed the presence of OutD (Figure 6A). In the absence of biotinylated PelB, OutD was not found among the proteins bound to streptavidin–agarose.

Interaction between OutD and PelB in vitro
The *in vivo* interaction between OutD and PelB may require additional proteins present in the periplasm or in the membrane fraction. We tried to reproduce this interaction *in vitro* with purified proteins by performing ligand blotting experiments. The outer membrane fraction of *E.coli* cells overproducing OutD was separated by SDS–PAGE and blotted onto a nitrocellulose membrane. The membrane was incubated with PelB. Binding of PelB to the membrane
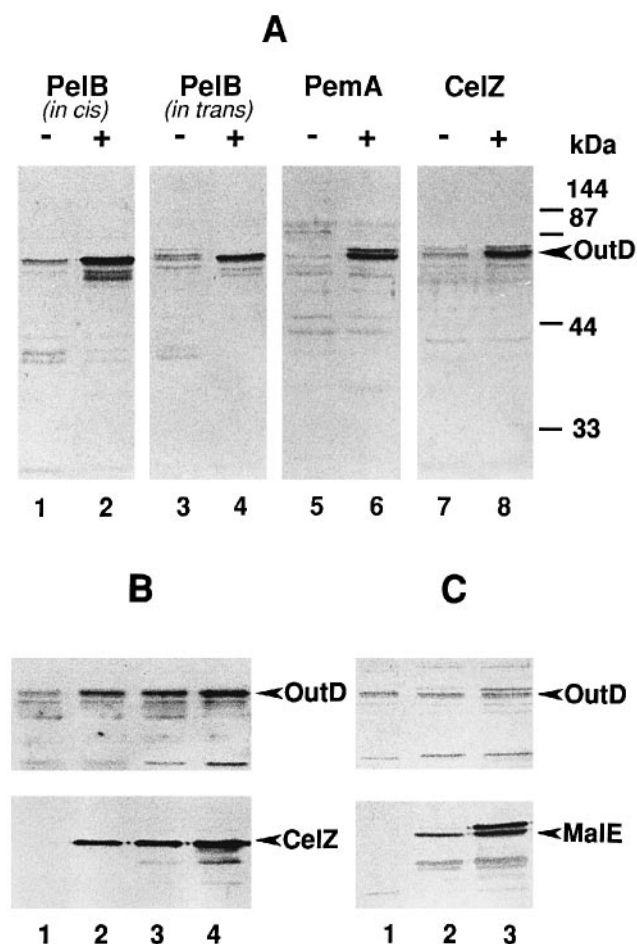
Fig. 4. Protection of OutD by the secreted proteins *in vivo*.
(**A**) Co-expression of *outD* with the genes encoding the secreted proteins (*pelB*, *pemA* and *celZ*) in *E.coli* NM522. Expression of *outD* alone, pTdB-OD (lanes 1 and 3) and pACOD (lanes 5 and 7); co-expression of *outD* and *pelB*, pTPLB-OD (lane 2) and pTdB-OD/pACPLB (lane 4); co-expression of *outD* and *celZ*, pACOD/pKCLZ (lane 8); and of *outD* and *pemA*, pACOD/pKME (lane 6). (**B**) Effect of the amount of EGZ on OutD protection. Expression of *outD* alone, pACOD (lane 1); co-expression of *outD* and *celZ*, pACOD/pSCLZ with *celZ* under the control of its own promoter (lane 2), pACOD/pKCLZ with *celZ* under the control of P*lac*: uninduced (lane 3) and induced with 1 mM IPTG (lane 4). (**C**) Effect of MalE overproduction on OutD. Expression of *outD* alone, pACOD (lane 1); co-expression of *outD* and *malE*, pACOD/pMAL-p2: uninduced (lane 2) and induced with 1 mM IPTG (lane 3). The culture samples were analyzed in SDS–PAGE followed by immunoblotting with anti-OutD and with anti-EGZ (B) or anti-pMalE (C).

was detected using anti-PelB antibodies followed by secondary antibodies coupled to peroxidase. The signal obtained was weak over a high background. To increase the sensitivity of this method, we used biotinylated PelB as a probe. Biotinylation of PelB was performed with the hydrophilic agent sulfo-NH-biotin, so that the biotinylation is restricted to the surface of the protein. The enzymatic and immunological properties of PelB appeared to be unchanged by the biotinylation. Thus, binding of PelB could be detected in a single step using peroxidase-conjugated streptavidin. Using this technique, we could detect the binding of PelB to OutD blotted onto a nitrocellulose membrane (Figure 7). However, an unidentified protein of a size almost identical to OutD (75 kDa), present even in *E.coli* cells that do not express OutD, was
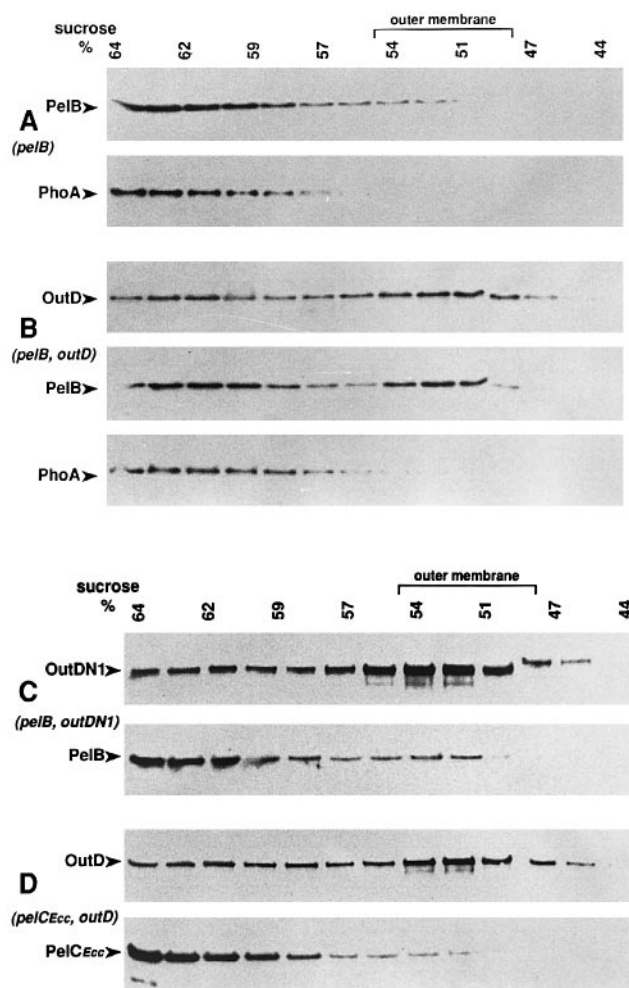
Fig. 5. PelB is associated with the outer membrane during *pelB–outD* co-expression in *E.coli*. Cell lysates of NM522(pTPLB) (PelB[+]) (**A**), NM522(pTPLB-OD/pACOS) (PelB[+], OutD[+], OutS[+]) (**B**), NM522(pODN1/pACPLB/pVLTS) (PelB[+], OutDN1^{+}, OutS[+]) (**C**) and NM522(pACTCc/pTdB-OD/pVLTS) (PelC$_{Ecc}$[+], OutD[+], OutS[+]) (**D**) were fractionated in a flotation sucrose gradient. Aliquots from each fraction were analyzed by SDS–PAGE and immunoblotting. The same nitrocellulose membrane was re-probed with anti-PelB, anti-OutD and anti-PhoA. The sucrose concentrations are indicated.

also able to bind PelB. Using anti-DnaK antibodies, we verified that this protein is not the general chaperone DnaK. When a ligand-blotting experiment was performed with biotinylated MalE, no interaction with OutD or with the 75 kDa protein was detected (data not shown). To confirm the specificity of the PelB–OutD interaction, we performed a chase experiment. Addition of an excess of unlabeled PelB to the incubation mixture strongly decreased the signal (Figure 7). Addition of a reducing agent dithiothreitol (DTT) to the incubation mixture prevented the specific binding of PelB to OutD (data not shown), confirming that, as previously suggested, a secreted protein has to be in a folded state to interact with the Out secretion machinery (Shevchik *et al.*, 1995).

Functional analysis of OutD deletion derivatives
To determine the regions of OutD involved in the interaction with OutS, with secreted proteins and in the formation of homomultimers, we constructed a series of OutD deletions (Figure 8). OutD is divided into two parts
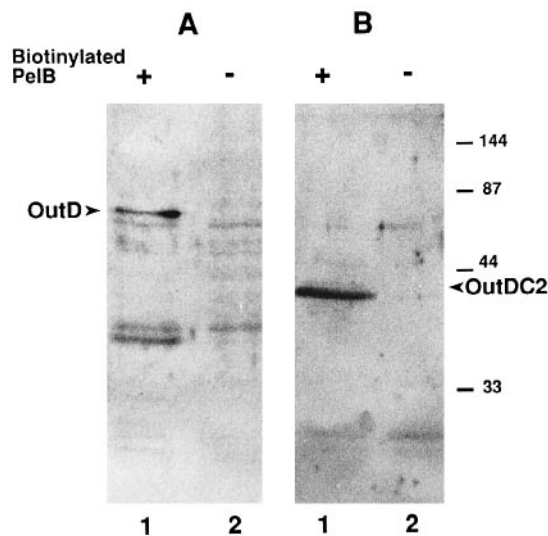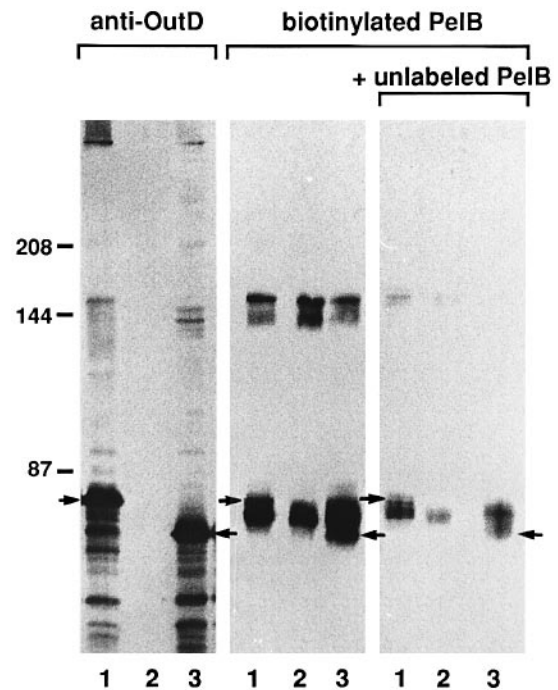
Fig. 6. Co-precipitation of OutD and OutDC2 with PelB. The outer membrane fractions of NM522(pTPLB-OD/pACOS) (PelB⁺, OutD⁺, OutS⁺) (**A**) and NM522(pTPLB-ODC2) (PelB⁺, OutDC2⁺) (**B**) were incubated with biotinylated PelB (lane 1) or with unlabeled PelB (lane 2). Proteins bound to streptavidin-conjugated agarose beads were analyzed by SDS–PAGE and immunoblotting with anti-OutD.

Fig. 7. Interaction of PelB with OutD *in vitro*. Proteins of the outer membrane fractions of *E.coli* BL21 (lane 2), BL21(pTdB-OD) overproducing OutD (lane 1) and BL21(pTdB-ODC1) overproducing OutDC1 (lane 3) were separated by SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with biotinylated PelB and, after washing, biotinylated PelB was revealed with streptavidin-conjugated peroxidase. Where indicated, a 10-fold excess of unlabeled PelB was added to biotinylated PelB during incubation. The position of OutD and OutDC1 was determined by immunodetection with anti-OutD on the same blot. The arrows indicate the positions of OutD and OutDC1.

by a 70 amino acid long stretch of serine and glycine residues (Condemine *et al.*, 1992) which could define two functional domains (Genin and Boucher, 1994). Two deletions of the C-terminal part of OutD were constructed. OutDC1 has a deletion of the last 62 amino acids; this fragment corresponds to the part of the protein that extends beyond the region common to all the GspD homologs (Martin *et al.*, 1993). OutDC2 is deleted of the last 328 amino acids of the protein; the deleted region is the part that is conserved in all the GspD homologs. Two deletions of the N-terminal part were also constructed. OutDN1 has a deletion of amino acids 66–116. OutDN2 has a deletion of the first 285 amino acids of OutD, placed under the control of the PelB signal sequence to allow its transloc-ation into the periplasm. This protein is very unstable and toxic for the cells. The same construct, without signal sequence (pODN), that accumulates in the cytoplasm, is stable, indicating that the periplasmic location makes it toxic.

Protection of the various OutD deletions by OutS was tested (Figure 2). This effect was visible with OutDN1 but not with OutDC1 or OutDC2. No increase in the amount of OutDN2 was visible. However, OutS seems to interact with OutDN2, since their co-production increases the toxicity of OutDN2. Thus, the interaction between OutD and OutS occurs through the C-terminal part of OutD.

The four OutD derivatives were tested for their ability to interact with PelB. Quantities of OutDC1 and OutDC2 detected in the cells were dramatically increased in the presence of PelB, while the amount of OutDN1 and OutDN2 was unchanged (Figure 9). Thus, the N-terminal part of OutD is involved in the protection effect of secreted proteins. To determine which region of OutD is required for the interaction with the secreted proteins, we tested by various approaches the ability of PelB to interact with the OutD derivatives. Analysis by sucrose density gradient of membrane fractions of cells co-expressing PelB and
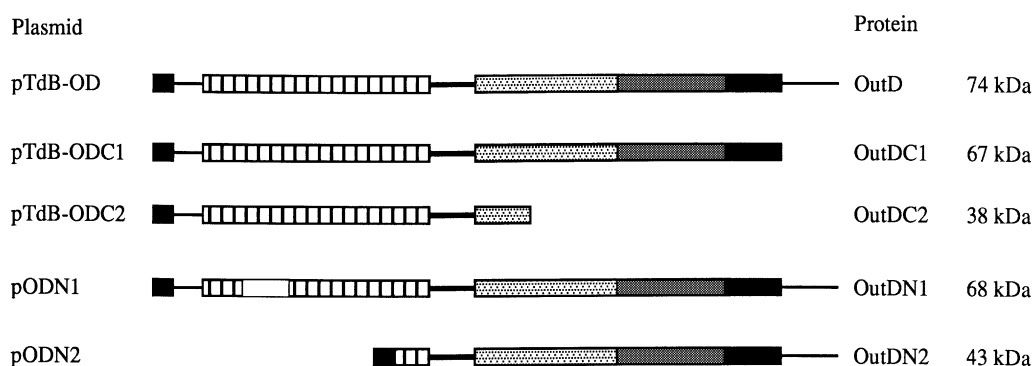
OutDN1 confirmed that these two proteins do not interact (Figure 5C). The amount of protein of derivatives that are not stabilized by OutS (OutDC1 and OutDC2) and of OutDN2 is too low to allow this type of analysis. However, the formation of complexes between OutDC2 and PelB was demonstrated by immunoprecipitation experiments (Figure 6B). Only OutDC1 was able to interact with PelB *in vitro* (Figure 7). OutDC1 was the only derivative that could form multimers in semi-denaturing conditions (Figure 1).

The ability of OutD and its derivatives to complement an *E.chrysanthemi outD* non-polar mutation and to inter-fere with the intact Out machinery in the wild-type background was tested. While the plasmid encoding the wild-type OutD (pTdB-OD) was capable of restoring the pectinase and cellulase secretion in strain A2591 (*outD⁻*), among the OutD derivatives, only the OutDC1-encoding plasmid (pTdB-ODC1) partially complemented the *outD* mutation (data not shown). The introduction of the OutD derivative-encoding plasmids into the wild-type strain A350 showed that OutDN1 exerted a negative dominance over the chromosomally encoded OutD, since it caused a decrease in the secretion efficiency (data not shown). No effect was observed with the other constructs.

PelC of E.carotovora does not interact with OutD of E.chrysanthemi
Despite a high level of homology, the secretion systems of *E.chrysanthemi* and *E.carotovora* are not interchang-

Plasmid   Protein

pTdB-OD   ■—▯▯▯▯▯▯▯▯▯▯▯—▨▨▨▨▨▨▨▨■—   OutD   74 kDa

pTdB-ODC1   ■—▯▯▯▯▯▯▯▯▯▯▯—▨▨▨▨▨▨▨▨■—   OutDC1   67 kDa

pTdB-ODC2   ■—▯▯▯▯▯▯▯▯▯▯▯—▨▨—   OutDC2   38 kDa

pODN1   ■—▯▯▯□□▯▯▯▯▯—▨▨▨▨▨▨▨▨■—   OutDN1   68 kDa

pODN2   ■▯▯—▨▨▨▨▨▨▨▨■—   OutDN2   43 kDa

Fig. 8. OutD and its deletion derivatives. OutD is divided into two parts by a serine- and glycine-rich region (solid black line): an N-terminal domain, variable among the OutD homologs (stripped box), and a conserved C-terminal domain (shaded boxes; shading represents the level of conservation). The black box represents a signal sequence. The open box shows the region deleted in OutDN1. The details of the construction are given in Table I.

able, and heterologously expressed Pels are not secreted (Lindeberg *et al.*, 1996). We wondered whether the limiting step in the secretion process could be the recognition of which protein to secrete by OutD. For this analysis, we used the *E.carotovora* PelC (PelC$_{Ecc}$) protein and the *E.chrysanthemi* OutD. PelC$_{Ecc}$ is a member of the neutral pectate lyases family and presents 76% homology with PelB$_{Ech}$ (Hinton *et al.*, 1989). Although a weak protection of OutD by PelC$_{Ecc}$ was observed *in vivo* when PelC$_{Ecc}$ was overexpressed, we could not detect a repositioning of PelC$_{Ecc}$ in the outer membrane by OutD$_{Ech}$ (Figure 5D), an interaction *in vitro* between the two proteins (data not shown) or a bacteriolytic effect of the co-expression of OutD and PelC$_{Ecc}$.

Discussion

Among the components of the Out machinery, OutD is the principal protein in the outer membrane. Thus, it would be expected to play a special role by controlling the traffic of secreted proteins. The results presented here focused on two features of OutD: its ability to multimerize in the outer membrane and its capacity to interact with proteins secreted by the Out secretion machinery. The presence of large amounts of OutD in the outer membrane requires OutS. In the absence of OutS, only a small amount of OutD can be detected in the bacteria. However, the relative amount of OutD associated with the outer membrane is the same in the presence or absence of OutS: in the absence of OutS, the augmentation of the amount of OutD due to PelB protection results in a corresponding increase in the quantity of OutD in the outer membrane (Figure 3). We have shown that the 62 C-terminal amino acids of OutD are necessary for its stabilization by OutS, since no protective effect by OutS was observed for either OutDC1 or OutDC2. However, even OutDC2, which lacks the 328 C-terminal amino acids of OutD, co-fractionated with the outer membrane fraction in a sucrose flotation gradient (data not shown). Thus, it seems that OutD can be associated with the outer membrane even in the absence of OutS.

Although it is obvious that OutS is required for the stabilization of OutD, its mechanism of action is not clearly understood. OutS could have two functions: (i) the protection of OutD while it crosses the periplasm. In the
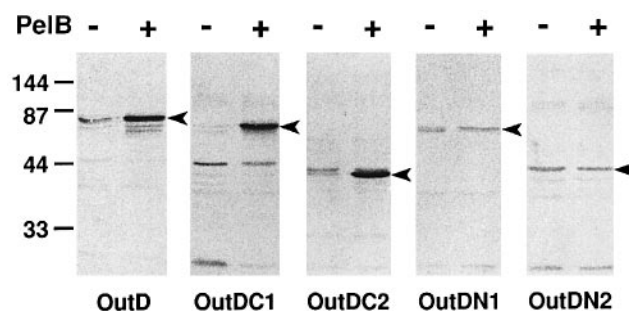

Fig. 9. The intact N-terminal part of OutD is important for its stabilization by PelB *in vivo*. Plasmids containing *outD* or its derivatives were introduced into strain NM522 carrying pACPLB (PelB$^+$) or pACYC184 (PelB$^-$). The amount of OutD and its derivatives in the cultures was estimated by immunoblotting with anti-OutD. The positions of OutD, its derivatives and the molecular mass standards are indicated.

absence of OutS, OutD would be degraded, only a small part of it escaping the degradation and becoming incorporated into the outer membrane. (ii) OutS could be necessary for the insertion of OutD in the outer membrane. In its absence, the improperly located OutD would be degraded. Further experiments will be required to determine the exact function of OutS.

A search for homology between the 62 C-terminal amino acids of OutD, which allow interaction with OutS, and other proteins detected only the C-terminal ends of *K.oxytoca* PulD and *E.carotovora* OutD, but no other GspD. These bacteria are the only two, apart from *E.chrysanthemi*, known to possess a GspS protein. The presence of a GspS and a C-terminal extension in GspD seems to be correlated. Insertion of GspD could occur without a GspS-like chaperone in bacteria, as in *P.aeruginosa* or *Xanthomonas campestris*.

Cross-linking data obtained with pIV, and confirmed by studies with PulD and XpsD, led Russel to suggest a channel-forming function for the GspD proteins (Kazmierczak *et al.*, 1994). However, chemical cross-linking sometimes allows for the formation of homomultimers of proteins that are not really assembled *in vivo* (Pugsley, 1996). We were able to demonstrate the existence of multimers, up to tetramers, of OutD *in vivo* without the use of cross-linking agents. The large size of OutD prevented the resolution of larger complexes by SDS–
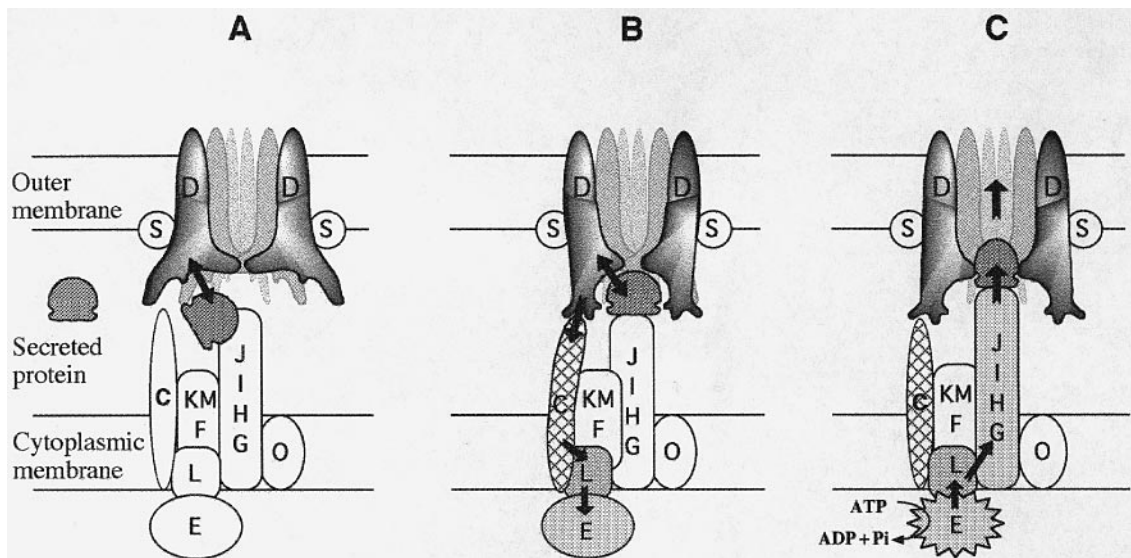
Fig. 10. Model of the *Erwinia chrysanthemi* Out secretion system. (**A**) OutD is incorporated via its C-terminal part into the outer membrane where, through the interaction of several molecules, it forms a gated channel. OutS probably remains associated with the OutD C-terminus. The N-terminal part of OutD is localized in the periplasm where it 'fishes' for the secreted proteins. (**B**) The interaction of secreted proteins with OutD induces its conformational change. This modification is transduced to the inner membrane part of the secretion machinery by one of the proteins containing a large periplasmic domain (OutC, K or M). OutC seems to be the most probable candidate to interact specifically with OutD and/or with the proteins to be secreted (Lindeberg *et al.*, 1996). This signal is transmitted, via this protein, to OutL and OutE located in the cytoplasmic membrane. (**C**) OutE, a putative ATPase (Sandkvist *et al.*, 1995), could energize other Out proteins, such as the pseudopilins, to push the secreted protein through the pore formed by OutD.

PAGE. Although we were not able to detect them, we cannot exclude the possibility that 10 to 12 mers are formed as observed for the pIV protein (Kazmierczak *et al.*, 1994). The formation of multimers was also observed with OutDC1, indicating that the C-terminal extension is not required for multimerization. Moreover, insertion of OutDC1 in the outer membrane, without the help of OutS, did not prevent its subsequent multimerization. The formation of a functional structure by OutDC1 was also confirmed by its ability to partially complement an *E.chrysanthemi outD* mutant. We could not show the formation of multimers with other OutD derivatives. It is possible that the complexes were formed but were then unstable during electrophoresis. It is also possible that they did not form, if the multimerization signal extends over several regions of OutD.

Besides its ability to multimerize, we showed that OutD is able to interact with proteins secreted by the Out machinery. Several techniques were used to demonstrate that, even in the absence of the other Out proteins, OutD is able to interact with secreted proteins *in vivo* and *in vitro*. The protection of OutD by secreted proteins is specific and stoichiometric, indicating the possible formation of a complex between OutD and these proteins. Co-fractionation of PelB and OutD in the outer membrane fraction showed that these complexes are stable. Unlabeled PelB could be chased from these complexes by biotinylated PelB, and co-precipitation of biotinylated PelB–OutD complexes could be observed. Taken together, these results indicate the existence of a specific interaction between OutD and secreted proteins in cells. The ligand-blotting experiments also demonstrated the possibility of a bicomponent, OutD–PelB, interaction. One question remains unanswered: are other cellular proteins involved in this interaction? It cannot be ruled out that additional protein(s),

i.e. other Out proteins, participate in this interaction *in vivo*, increasing its affinity and stability. Another important feature of the OutD–secreted proteins interaction, determined by ligand-blotting experiments, is that only correctly folded secreted proteins are able to interact with OutD.

The *in vivo* experiments on the protection of the OutD derivatives by PelB showed that an intact N-terminal region of OutD is necessary for the interaction with secreted proteins. The OutD N-terminus seems to be exposed to the periplasm, since ~50% of OutDC2 is liberated from cells, together with other periplasmic proteins, by osmotic shock. Its function could be to 'fish' for the secreted proteins in the periplasm (Figure 10A). The 50 amino acid N-terminal deletion of OutDN1 prevents its interaction with the secreted proteins, since no protection was observed *in vivo* (Figure 9). Thus, the negative dominance of OutDN1 over the wild-type OutD, observed in A350(pODN1), could be the result of the formation of mixed multimers in the outer membrane, unable to recognize the secreted proteins.

Another function of the N-terminus of OutD could be to gate the pore it is forming. The interaction of the secreted proteins with OutD could provoke a modification of the structure of the channel: co-expression of *pelB* and *outD* in *E.coli* at 37°C provokes cell lysis. This effect does not result from the overproduction of OutD, since co-expression of *outS* and *outD* did not produce that phenotype. This bacteriolytic effect could result from a partial opening of the channels formed by OutD, due to the interaction of OutD with the secreted proteins. The toxic effect of OutDN2, which lacks the N-terminal domain of OutD, could result from the absence of this gating activity. Co-expression of OutC, K, L and M together with OutD and PelB did not prevent the bacterio-

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Table I. Plasmids used in this study

Plasmid	Relevant characteristics	Reference
pGP1-2	Km^R, the T7 RNA polymerase gene under control of the λP$_L$ promoter and the *c*I857 repressor	Tabor and Richardson (1985)
pT7-6	Ap^R, the φ10 promoter	Tabor and Richardson (1985)
pTPLB	pT7-6 with a *Nsi*I–*Bam*HI (1635 bp) *pelB* fragment	this work
pTPLB-OD	pTPLB with a *Pst*I–*Xma*I (2571 bp) *outD* fragment inserted into the *Bam*HI site downstream of the *pelC* promoter	this work
pTdB-OD	pTPLB-OD with a *Hin*dIII 1064 bp *pelB* deletion	this work
pTdB-ODC1	pTdB-OD with a *Nru*I–*Sma*I (247 bp) deletion (OutDΔ648–710)	this work
pTdB-ODC2	pTdB-OD with an *Eco*RV–*Sma*I (1047 bp) deletion (OutDΔ382–710)	this work
pODN1	pBluescript KS Ap^R (Stratagene) with the *Sal*I–*Cla*I *outD* fragment with a *Avi*II–*Msc*I (147 bp) deletion (OutDΔ66–116)	this work
pODN	pKSM717 Ap^R (Maneewannakul *et al.*, 1994) with the *Sca*I–*Sac*I *outD* fragment under control of the ATG start codon after P*lacUV5* (OutDΔ1–285)	this work
pODN2	pET-20b(+) Ap^R (Novagene) with the *Nco*I–*Eco*RI fragment from pODN, inserted under control of the *pelB* signal sequence	this work
pACOD	pACYC184 Cm^R with the *Sal*I–*Cla*I (4840 bp) *outC–outD* fragment	this work
pACPLB	pACYC184 with the *Sph*I–*Bam*HI (1635 bp) *pelB* fragment	this work
pACOS	pACYC184 with a 1070 bp *outS* fragment	this work
pVLTS	pVLT31 Tc^R (de Lorenzo *et al.*, 1993) carrying *outS* under control of the *tac* promoter	this work
pKCLZ	pBluescript KS Ap^R with the *Hin*dIII–*Pvu*II (2570 bp) fragment from pSR1662 (Reverchon *et al.*, 1994) with *celZ* under the control of P*lac*	this work
pSCLZ	As in pKCLZ, in the opposite orientation in pBluescript SK Ap^R	this work
pKME	pBluescript KS Ap^R with *pemA* subcloned from pPME3 (Shevchik *et al.*, 1996)	this work
pJS6197	pUC19 carrying the *E.carotovora pelC* gene	Hinton *et al.* (1989)
pTPLCc	pT7-5 with the *E.carotovora pelC* gene	this work
pACTCc	pACT3 Cm^R (Dykxhoorn *et al.*, 1996) carrying the *E.carotovora pelC* gene under control of the *tac* promoter	this work
pACOC	pACYC184 carrying *outC*	this work
pACOG-M	pACYC184 carrying *outG-M*	this work
pMAL-p2	Ap^R with the *malE* gene under control of the P*tac* promoter	Biolabs

lytic effect, suggesting that the gating activity of OutD is not modulated by the presence of other Out proteins. However, it cannot be ruled out that one of these proteins interacts with the OutD–PelB complex without suppressing the bacteriolytic effect. Moreover, the secretion process is very dependent on the stoichiometry of the different Gsp proteins (Pugsley, 1996).

Among members of the GspD superfamily, the N-terminal region of proteins involved in the same type of export mechanism share some homology. However, within each family, the differences in this domain could determine the specificity of the protein to be secreted. Despite the high sequence similarity of *E.carotovora* and *E.chrysanthemi* OutD proteins, which possess 72% amino acid identity, they are not interchangeable (Lindeberg *et al.*, 1996). We have shown that the OutD$_{Ech}$ is unable to bind PelC$_{Ecc}$, although the homology between PelB$_{Ech}$ and PelC$_{Ecc}$ is higher than between PelB$_{Ech}$ and the other Pels secreted by *E.chrysanthemi*. All these data point to the recognition of the secreted proteins by GspD as the key to the specificity of the secretion machinery. The construction of hybrids between different GspD proteins, particularly exchange of their N-terminal regions, will confirm if the secretion specificity is only determined by this protein.

The interaction of secreted proteins with OutD is not sufficient to trigger their secretion in *E.coli*. This function requires the other Out proteins. We propose that this interaction modifies the conformation of OutD and that this modification is transduced to the inner membrane part of the secretion machinery by one of the proteins possessing a large periplasmic domain (OutC, K or M). This signal would be transduced to OutE which could then energize an Out protein to open the pore formed by OutD and push the secreted protein into the outer medium (Figure 10). Such a mechanism would insure that the pore is open only when a protein is ready to be secreted.

Materials and methods

Bacteria and plasmids
Strains of *E.coli* used in this study are NM522 [*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5 (r$_k^-$ m$_k^-$) (F′*proAB lacI^q* Δ*lacZM15*)] (Stratagene); K-38 [HfrC(λ) *phoA4 pit-10 tonA22 ompF627 relA1*] (Russel and Model, 1984); and BL21(DE3) (F$^-$ *dcm$^-$ ompT* r$_B$- m$_B$- lon$^-$λ) (Stratagene). *Erwinia chrysanthemi* 3937 derivatives were A350 (*lmrT^c lacZ*) (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1985) and A2591 (*lmrT^c lacZ* Δ*outD*), obtained by recombination into the A350 chromosome of an *Avi*II–*Msc*I in-frame deletion of *outD*.

Plasmids used in this study are listed in Table I. pTPLB-OD and its derivatives carry the *outD* gene under control of the *pelC* promoter, allowing its high level constant expression in *E.coli* and galacturonic acid-inducible expression in *E.chrysanthemi*.

Growth conditions
Erwinia chrysanthemi cells were usually grown at 30°C and *E.coli* at 37°C in Luria–Bertani (LB) medium supplemented, when required, with sodium polygalacturonate and galacturonate (0.2%). Antibiotics were added to the following concentrations: ampicillin 150 μg/ml, chloramphenicol 25 μg/ml and kanamycin 50 μg/ml. Plate assays for the detection of pectinase and cellulase activities were performed as described previously (Condemine *et al.*, 1992).

Protein production, purification and labeling
OutD protein was overproduced in the K38(pGP1-2/pTdB-OD) strain and partially purified from the outer membrane (Triton/Mg-insoluble fraction), as described previously (Shevchik *et al.*, 1996).

To overproduce PelB, BL21(pTPLB) cells were grown at 30°C in LB containing 150 μg/ml ampicillin. At an OD$_{600}$ of 1.0, IPTG was added to 1 mM and, after 2 h of additional growth, the cells were harvested by centrifugation for 5 min at 5000 *g* at 4°C and frozen at –80°C. The

overproduced protein was extracted from cells by three cycles of freezing–thawing (Johnson and Hecht, 1994). PelB was concentrated from the extract by 40–60% ammonium sulfate precipitation. The pellet was solubilized in 10 mM sodium phosphate buffer (NaPB) containing 1.2 M ammonium sulfate and loaded onto a Phenyl-TSK-GEL column, equilibrated with the same buffer. PelB was eluted with a 1.2–0.8 M ammonium sulfate linear gradient, concentrated with Centricon 10 (Amicon) and applied to a Superdex 200 (Pharmacia) gel filtration column. The PelB peak fractions were pooled and concentrated with Centricon 10 in 20 mM NaPB pH 7.0, 1 mM $CaCl_2$ (buffer A). No contaminating protein could be detected in this preparation by loading 20 μg of protein onto SDS–PAGE. Biotinylation of PelB was performed with sulfo-NHS-biotin (Pierce). The protein (5 mg/ml) was incubated with sulfo-NHS-biotin (1 mg/ml) in buffer A for 1 h at 4°C, then washed 10 times in the same buffer with Centricon 10. Glycerol was added to 50% and the preparation was stored at –20°C.

PelC$_{Ecc}$ was overproduced in BL21(pTPLCc) cells. The same conditions of overproduction and the same scheme of purification and biotinylation as for PelB were used. Higher concentrations of ammonium sulfate were used during purification: 80% for the protein precipitation, 1.7 M for the loading onto a Phenyl-TSK-GEL column and 1.7–1.2 M for the elution linear gradient.

The 'Protein fusion and purification system' (Biolabs) was used for the maltose-binding protein (MalE) overproduction and purification. The same biotinylation conditions as for PelB were used.

Subcellular fractionation

Exponentially grown cultures (OD$_{600}$ 0.8–1.0) were usually used for the cell fractionation. Release of periplasmic proteins was performed by osmotic shock (Copeland *et al.*, 1982). The cell membrane fractionation was performed by sucrose gradient centrifugation, in flotation or sedimentation gradients, in a Sorvall AH650 rotor for 48 h at 48 000 r.p.m. at 8°C, as described previously (Shevchik *et al.*, 1996). For the OutD purification, membrane fractionation was performed by detergent extraction (Schnaitman, 1971).

Co-precipitation of OutD and PelB was performed using the OutD–PelB-containing outer membrane fractions from the sucrose gradient centrifugation (55–50% sucrose). Three hundred μl of the sample were diluted to 1.3 ml in 10 mM HEPES pH 7.5 and incubated for 1 h with biotinylated or unlabeled PelB (4 μg). Then streptavidin-conjugated agarose beads (BRL) (~30 μl) were added and incubated for an additional 30 min. All incubations were performed at 4°C with gentle agitation. After washing three times for 5 min with the same buffer, the agarose beads were mixed with 30 μl of SDS–PAGE sample buffer, incubated for 5 min at 30°C, and the resulting supernatant was analyzed by SDS–PAGE and immunoblotting with anti-OutD antibodies.

Gel electrophoresis, immunoblotting and ligand blotting

SDS–PAGE was usually performed according to Laemmli (1970). Concentrations of acrylamide and bisacrylamide varied from 8 to 15% and from 0.2 to 0.4% respectively, depending on the experiment. Proteins were transferred onto nitrocellulose in a semi-dry apparatus and the membrane was incubated with antibodies and developed with the ECL detection kit (Amersham), as described previously (Shevchik *et al.*, 1996). The primary antibodies used were anti-PelB diluted 1:5000 (Shevchik *et al.*, 1995), anti-PhoA diluted 1:10 000 (5 Prime→3 Prime, Inc.), anti-EGZ diluted 1:5000 (provided by F.Barras), anti-DnaK diluted 1:5000 (provided by G.Richarme) and anti-OutD diluted 1:3000 (the antiserum was raised against OutD purified from an *E.coli* recombinant strain). To re-probe the same nitrocellulose membrane with different antibodies, it was treated according to the ECL protocol.

For the ligand-blotting experiments, the OutD-containing outer membrane fraction was separated by SDS–PAGE and blotted onto nitrocellulose. The membrane was saturated with 3% bovine serum albumin and then successively incubated for 1 h with biotinylated PelB (0.5 μg/ml), washed three times for 10 min, incubated for 1 h with horseradish peroxidase-conjugated streptavidin (Pierce) (40 ng/ml) and washed three times for 10 min prior to developing with ECL. The incubations were usually performed at room temperature, with agitation, in 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20.

Acknowledgements

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